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DEVELOPMENT OF A SYSTEM TO STUDY GENE ACTIVATION IN MAMMALIAN CELLS TREATED WITH BIS (2-CHLOROETHYL)SULFIDE

Final Report

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We have shown that treatment of S49 mouse lymphoma cells with sulfur mustard (SM) results in about a 150-fold increase in the frequency of cadmium-resistant (Cd^r) variants compared to untreated controls (1). We have examined 18 of these Cd^r variants in detail as well as 5 spontaneous variants, and we find that virtually all of them make metallothionein I (MT-I), metallothionein II (MT-II), or both. Curiously, the spontaneous Cd^r variants make only MT-II, while the Cd^r variants from sulfur-mustard-treated cells often synthesize both RNAs. In a series of genomic blotting experiments (southern blots), we found that most of these MT+ lines showed no major amplifications, gene rearrangements, or deletions, thus ruling out such changes as the major cause for the activation of these genes. Analysis of DNA-methylation patterns by Hpa II digestion followed by genomic blotting revealed a complex pattern of demethylation in MT+ variants, but no consistent pattern. These data suggest that there are no crucial sites at which demethylation must occur in order for variants to express MT. In other experiments, we determined that treatment with half-sulfur mustard (2-chloroethyl-

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Abstract continued

sulfide; HSM) also resulted in the appearance of Cd^{r}_{4} variants. At the highest doses tested, treatment with either agent resulted in a several-hundred-fold increase in the number of Cd^{r}_{4} variants. These data indicate that DNA cross-linking is not necessary for the induction of Cd^{r}_{4} variants by the mustards, and suggest (by extension) that the activation of MTs is not dependent on cross-linking.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

SUMMARY

In the first phase of this study we examined the activation of two quiescent genes, metallothionein I (MT-I) and metallothionein II (MT-II), in S49 mouse thymic lymphoma cells by ethylnitrosourea (ENU) and N-acetoxy-2-acetylaminofluorene (NA-AAF). We found that 1-3 mM ENU and 5-15 μM NA-AAF were moderately toxic to cells and produced large (~10-30-fold) increases in the number of cadmium-resistant variants. About 65% of these cadmium-resistant variants made either MT-I, MT-II, or both RNAs by dot hybridization, and northern blotting confirmed that the transcripts were of the appropriate size. Quantitative dot hybridization indicated that different cell lines had different constitutive levels of MT expression and different degrees of cadmium inducibility. Survival studies and mixing experiments indicated that cadmium-resistant variants are induced by these alkylating agents and not selected by them from previously existing variants in the initial cell population. Analysis of DNA methylation patterns by digestion with HpaII endonuclease and Southern blotting indicated that demethylation was associated with the activation of these genes, but no simple methylation pattern was apparent. Using restriction endonuclease digestion and Southern blotting, we have not detected any insertions, deletions, rearrangements, or amplifications associated with the activation of either MT gene. All of these data demonstrate that alkylating agents can activate quiescent genes in mammalian cells.

In the second phase of these experiments, we have examined sulfur mustard (SM) and half mustard (HSM). We established a range of toxicity for SM in S49 cells (*e.g.*, 0.5 μM SM treatment yields a 20% survival) and found that per survivor there is as much as a 140-fold increase in Cd^r variants. We have examined 18 of these Cd^r variants in detail as well as 5 spontaneous variants and find that virtually all of them make metallothionein I (MT-I), metallothionein II (MT-II) or both. Curiously, the spontaneous Cd^r variants make only MT-II while the Cd^r variants from sulfur mustard treated cells often synthesize both RNAs. In a

series of genomic blotting experiments (Southern blots), we found that most of these MT^+ lines showed no major amplifications, gene rearrangements, or deletions, thus ruling out such changes as the major cause for the activation of these genes. Analysis of DNA methylation patterns by HpaII digestion followed by genomic blotting revealed a complex pattern of demethylation in MT^+ variants, but no consistent pattern. These data suggest that there are no crucial sites at which demethylation must occur in order for variants to express MT. In other experiments, we determined that treatment with half sulfur mustard (2-chloroethyl-ethyl sulfide; HSM) also resulted in the appearance of Cd^r variants. These data indicate that DNA cross-linking is not necessary for the induction of Cd^r variants by the mustards and suggest (by extension) that the activation of MTs is not dependent on cross-linking. Thus it appears that an unrecognized and potentially important action of SM and HSM is the activation of quiescent genes.

INTRODUCTION

To date, most analyses of monofunctional and bifunctional alkylating agents have focused on properties such as cell killing and mutation, which are clearly deleterious to cells in culture and intact animals (1-3). However, administration of chemical carcinogens to animals results in the production of tumors which display new phenotypic properties (4,5). A substantial portion of such agents are alkylating agents or aryl alkylating agents (2). Although the role of mutation in neoplasia has received prominent attention recently (6), other mechanisms may also be involved. One possibility is that damaging agents themselves can activate quiescent (unexpressed) genes. While it is likely that during the neoplastic process the potential targets for such reactions are not specific structural (*e.g.*, actin or tubulin) or functional (*e.g.*, enzymes) genes but rather regulatory genes, nevertheless such differentiated gene products are more accessible to study. It is also well known that monofunctional and bifunctional agents can affect development (*e.g.*, 7). It is unclear, however, if such changes can result from inappropriate gene activation. By analogy with carcinogenesis it is possible that toxic agents may cause inappropriate gene expression during development. In order to study this important aspect of molecular toxicology, a practical approach is to use genes whose expression can be selected for in culture.

Several years ago, we demonstrated that ultraviolet radiation could activate quiescent metallothionein genes in cultured mouse thymic lymphoma cells (S49) (8). The metallothioneins are a family of low molecular weight proteins that protect cells against heavy metal toxicity and may be involved in zinc regulation as well (9). Although most cells make metallothioneins constitutively, mouse thymus and S49 thymic lymphoma cells do not (10,11). In the mouse there are two metallothionein genes, MT-I and MT-II, that are closely linked and show approximately 75% amino acid homology (12). Thus this small family of unexpressed genes in mouse thymic lymphoma cells represents a useful system in which to explore the

activation of quiescent genes. One may take advantage of the toxicity of cadmium to S49 cells to select for variants which become cadmium-resistant. These resistant variants may then be analyzed on a comparative basis with S49 cells for the expression of metallothioneins.

In this report we summarize our findings with two alkylating agents [N-acetoxy-2-acetylaminofluorene (NA-AAF) and ethylnitrosourea (ENU)], sulfur mustard (SM) and half sulfur mustard (HSM). All four agents induce the conversion of Cd^s (cadmium-sensitive) S49 cells to Cd^r (cadmium-resistent) variants. In most instances variants synthesize metallothionein I (MT-I) RNA, MT-II RNA, or both. The mechanism underlying this change is unclear in that it is not associated with amplifications or major deletions or rearrangements of the S49 genome. However, the results demonstrate that three agents can activate the expression of quiescent MT genes and suggest that activation of unexpressed genes is a potentially important and heretofore unrecognized action of these and similar agents.

MATERIALS AND METHODS

Cell culture. Rat thymic lymphoma cells (S49) were grown in Ham's F12 medium with 10% fetal calf serum, 2X bicarbonate and penicillin and streptomycin. To select for cadmium-resistant variants, cells were treated with the indicated concentrations of ENU or NA-AAF (dissolved in 1% DMSO) for 3 hr and then placed in liquid medium for 0-5 days. For sulfur mustard (2-chloroethyl-ethyl sulfide; Aldrich) and half mustard experiments SM or HSM diluted in absolute ethanol was added at the indicated final concentrations (see Figures and Tables) by diluting a small amount of absolute ethanol containing SM (approximately 10-25 µl) in 30 ml of medium containing approximately 1×10^6 S49 cells/ml. Procedures were done in screw cap 50 ml plastic centrifuge tubes in a chemical fume hood. After 3 hours incubation at 37°C, the cells were spun down and resuspended in fresh medium and maintained in fresh medium for 0-5 days.

We used growth in soft agar to determine colony-forming efficiency (CFE) in the

presence or absence of Cd and to isolate cadmium-resistant variants. For CFE, cells were plated 2-3 x 10^2 in 100 mm petri dishes with a bottom layer which contained 10 ml of complete medium with 0.5% bacto agar with or without 5 μm $CdSO_4$. The top layer was composed of complete medium containing 0.5% low melt agar with or without Cd. The final volume of the top agar was 1 ml which contained the cells. For selecting cadmium-resistant variants in soft agar, 0.5 x 10^6-1.0 x 10^6 cells per 100 ml petri dish were used. Usually 5 plates were used per point. Cadmium-resistant variants were propagated in medium containing 5 μm cadmium while S49 cells were grown in cadmium-free medium. Small variations on these techniques were used for analysis of induction versus selection (see Tables) and analysis of potential selective growth advantage of Cd^r cells following carcinogen treatment (see Tables). Plates were incubated for 8-14 days, and the colonies were counted.

Nucleic acid procedures. Total RNA was isolated by guanidinium isothiocyanate/cesium chloride centrifugation (13). In some instances DNA was isolated from the same gradients or in other cases by phenol extraction, RNAse digestion and ethanol precipitation (13). In NA-AAF experiments MT-I and MT-II RNA levels were measured by dot hybridization (14). 0.1-5 μg of total RNA was used, and the probes were single stranded M13 phage DNAs containing full length MT-I cDNA or MT-II cDNA consisting of the last two exons of the gene (gifts from R. Palmiter; ref. 12). Labeling was performed by incubating DNA, α^{32}P dCTP or dATP (Amersham or New England Nuclear, 3000 Ci/mmol) and unlabeled nucleotides with the Kenow fragment of DNA polymerase I (New England Biolabs) and an M13 primer which hybridized 5' to the cDNA insert (P.L. Biochemicals) (15). Thus the probe was partially double-stranded and was stabilized by UV irradiation following incubation with 4,5,8-trimethylpsoralen (Sigma) (16). Hybridization and washing were done according to the method of Thomas (17).

To calculate the number of molecules/cell we cloned the MT-I and MT-II cDNA probes

into pSP6 and transcribed the RNA from the SP6 promoter using SP6 polymerase (Promega Biotech) according to the manufacturer's description. We then quantitated the amount of transcribed RNA by absorbance (260 nm) measurements, blotted it as standards along with cellular RNA and calculated the number of metallothionein molecules/picogram cellular RNA by scanning densitometry of standards and cell spots. By knowing the number of picograms of RNA/cell (~12), one can calculate the number of metallothionein molecules per cell. For Southern blotting and northern blotting involving Cd^r clones derived from SM treatment, the procedure was as follows. Restriction endonucleases were purchased from standard suppliers (*e.g.,* Bethesda Research Laboratories, Amersham, New England Biolabs) and used as suggested by the manufacturer. Southern blots were performed in the standard fashion using ^{32}P nick-translated probes (13). Probes were made by isolating fragments from pBR322 plasmids containing pieces of the MT-I or MT-II genome (Figure 2, ref. 12,18,19). Northern blots were performed by standard procedures using 10 ug total RNA and a 335 bp cDNA for MT-I and a 210 bp probe from the 3' end of MT-II (12,13,18,19).

For initial studies of DNA methylation, DNA (10 μg) was digested with restriction endonucleases (HpaII or MspI, Bethesda Research Labs) according to the manufacturer's directions. Digests were electrophoresed on 1% agarose gels, transferred to nitrocellulose and hybridized to nick-translated fragments from the 5' end of each gene (8). For the MT-I gene region we used R600, a 600 bp fragment including approximately 550 bp 5' to the transcription start and approximately 60 bp of the transcribed region (Figure 1 and ref. 8), and PP300, a 300 bp fragment running from PvuII to PstI in the non-coding portion of MT-I (Figure 1). For MT-II a 1500 bp fragment from the 5' region of MT-II running between 2 Nco I sites (Figure 1 and ref. 12) was used.

RESULTS

Toxicity of ENU and NA-AAF. We examined the toxicity of ENU and NA-AAF by treating S49

cells in complete medium for 3 hr with either ENU or NA-AAF; following treatment, 200-300 cells were plated in soft agar. The data in Table 1 indicate that 1-3 mM ENU and 5-15 μM NA-AAF are workable ranges for studying the appearance of cadmium-resistant variants in S49 cells.

Treatment of S49 Cells with ENU and NA-AAF Results in Increased Numbers of Cd^r Variants. When cells are treated with these same concentrations of alkylating agent, allowed a five day recovery/expression period and then plated in soft agar containing 5 μM cadmium, a large increase in the number of cadmium-resistant variants is seen following treatment (Table 1). The spontaneous level of cadmium-resistant variants in this population is about 30 per million cells; treatment with ENU results in a 9-27 fold increase in cadmium-resistant variants at 1 and 3 mM ENU, respectively. Similarly, treatment with NA-AAF resulted in a 13-fold increase in cadmium-resistant variants at 15 μM NA-AAF. At similar levels of toxicity (ENU at 2 mM and NA-AAF at 15 μM), similar increases in the number of cadmium-resistant variants occurred, 17-fold for ENU and 13-fold for NA-AAF (Table 1). Since a 5 day recovery period resulted in almost a 30-fold increase in the number of Cd^r variants (at 3 mM ENU), we did not examine shorter expression times. When UV was used in an examination of expression times we found that there was little difference between 3 and 5 days (Lieberman, unpublished).

Cd^r Variants of S49 Cells Synthesize MT-I and MT-II. We isolated and propagated 47 cadmium-resistant variants obtained from treatment of cells with ENU and NA-AAF. Total RNA was isolated from these cells and hybridized to ^{32}P-labeled probes for MT-I and MT-II by dot hybridization (see Materials and Methods). We found that about two-thirds of these cells synthesized at least one metallothionein RNA (Table 2). Thus, not only does treatment of S49 cells with alkylating agents result in the appearance of cadmium-resistant variants, most of these variants also synthesize metallothionein RNAs. What is surprising about these results is the large number of variants which make both MT-I and MT-II: Of 31 MT^+ variants, 15 or almost 50% make both MT-I and MT-II RNA. Thus there appears to be coordinate activation

of these two closely linked genes following treatment with alkylating agents. These results are interesting from another perspective: Normally, cells and tissues synthesize both metallothioneins simultaneously (*e.g.,* 12); thus, the appearance of lines which make only one metallothionein is unusual.

To confirm the dot blot results, we also did selective northern blot analysis of some cadmium-resistant variants. These results (Figure 2) demonstrate that cadmium-resistant variants derived by treatment of S49 cells with alkylating agents make metallothionein RNA transcripts of the appropriate size and are capable of synthesizing MT-I alone (lines 8 and 9), MT-II alone (lines 17 and 18) and MT-I and MT-II (lines 12 and 13).

We examined the regulation of MT RNA synthesis in some of these lines. Because both cadmium and dexamethasone are known to augment metallothionein synthesis in both cultured cells and in vivo (*e.g.,* 10,11), we placed cells in cadmium-free, dexamethasone-free medium for several weeks and treated cells with either phosphate-buffered saline, 100 nM dexamethasone or 20 μM Cd for 6 hr prior to harvest. We performed quantitative dot blotting using SP6 synthesized RNA as a standard for quantitation (Materials and Methods). We found that the MT^+/Cd^r variants fell into a number of categories (Table 3). Some lines like ENU-5 or AAF-1 make only a single MT species. Synthesis of MT is highly inducible with cadmium and only slightly responsive, if at all, to dexamethasone. Another line (ENU-10) showed high constitutive levels of both MT-I and MT-II and only a slight inducibility by cadmium. Other lines like ENU-7 and AAF-6 synthesized constitutively low levels of MT-I and MT-II, and in these lines both genes could be induced by Cd. ENU-6 shows a divergence of regulation: MT-I is not Cd-inducible while MT-II shows a 7-fold increase after Cd addition.

Cd^r Variants in S49 Cells are Induced by ENU and NA-AAF. We have examined the question of whether ENU and NA-AAF induce the appearance of new variants or select previously existing variants from the treated populations. Formally, variants may arise either by de novo

events which generate new variants or selection of previously existing variants in the cell population, presumably by selective growth advantage of the variants. To analyze this problem, we did an experiment in which we plated untreated S49 cells or treated S49 cells with 15 μM NA-AAF or 2 mM ENU and plated them immediately after damage; we measured the relative colony-forming efficiency (CFE) in soft agar either with or without cadmium. We then computed the number of cadmium-resistant variants per 10^6 viable cells. The argument is that if these agents are selecting for cadmium-resistant variants then we should see increased numbers of cadmium-resistant variants per survivor in groups plated immediately after damage. In fact, we find the same number of variants in these experiments (Table 4). Thus these agents must induce the cadmium-resistant phenotype rather than select for it.

As a further check on the validity of this conclusion, we also examined the growth rate of cadmium-resistant and cadmium-sensitive cells following carcinogen treatment. The rationale for these experiments is that if the conclusion that these agents induce rather than select for the cadmium-resistant phenotype is correct, then growth rates of cadmium-resistant cells and cadmium-sensitive cells should be similar following carcinogen treatment. In fact, this is what we found (Table 5). We mixed S49 cells and either an ENU or an NA-AAF derived cadmium-resistant line and divided each cell population into two groups: The first received no treatment and was plated and seeded in soft agar with or without cadmium while the second was treated with either 2 mM ENU or 10 mM NA-AAF and was plated under similar conditions. We found that cadmium-resistant cells grew no better than control cells following carcinogen treatment; thus cadmium resistance does not supply a selective advantage for growth of S49 cells either before or after carcinogen treatment. These results provide added evidence that the cadmium-resistant phenotype is not selected for by these agents, but rather is induced by it.

Demethylation of the MT Locus is Associated with Activation of MT Genes. Since the activation of many genes is associated with demethylation (loss of 5-methylcytosine moieties)

in the body of the gene and/or the 5' flanking region (20), we examined methylation changes in lines in which MT-I and MT-II had been activated. We cut DNA from these lines with HpaII, a restriction endonuclease which cleaves CCGG regions only when the internal C is unmethylated. Many HpaII sites have been identified in the MT gene region (Figure 1). Southern blotting and probing with MT-I or MT-II probes revealed that wild-type S49 cells (*i.e.*, those which are Cd-sensitive) showed no cutting in the MT-I gene region and one cut in the MT-II region (Figure 3); these results indicate that, like other inactive genes, the metallothionein genes are highly methylated. In lines expressing MT-I there is a ~1.0 kb band which is diagnostic of expression. This band comes from the loss of methyl groups at two sites in the immediate 5' region of MT-I; a similar band, ~0.7 kb in length, is present in lines which make MT-II and comes from demethylation in the 5' region of MT-II. In addition, variable numbers of bands of higher molecular weight are seen, indicating demethylation at other sites in the metallothionein gene region. What makes interpretation of these data difficult is that all of these bands are of variable intensity; the most likely explanation for this is heterogeneity in the cell population with respect to methylation status. This heterogeneity may result from either the acquisition of additional unmethylated sites or the remethylation of existing unmethylated sites subsequent to the generation of the initial MT^+ cells by NA-AAF or ENU. Thus correlating methylation changes precisely with expression is not possible in this system; however, these results may indicate that demethylation at individual sites is not crucial for gene activation, but rather that demethylation within a region is important (19).

Analysis of the Metallothionein Gene Region in MT^+ Variants. To determine if activation of the metallothionein genes was related to changes in genome structure, we analyzed 31 variants which were either MT-I^+, MT-II^+, or MT-I^+/MT-II^+. Variants were generated by treatment with NA-AAF or ENU and represent the same cell lines used in other experiments (*e.g.*, Table 3, Figs. 2 and 3). We used three probes which span the metallothionein locus

(Figure 1) and in separate experiments digested these variant lines with EcoRI, XbaI, PstI or BamHI. By a combination of these restriction endonucleases and probes we were able to generate eight different fragments which spanned approximately 25 kb of the mouse metallothionein locus from approximately 10 kb 5' of the MT-II gene (the 5' gene) to approximately 2 kb 3' of the MT-I gene (Table 6). When all of these variants were compared with parental (untreated, cadmium-sensitive) S49 cells, we found no new bands and no changes in the size of existing bands. This finding indicates that there were no detectable deletions, insertions, or rearrangements using this method. There were no changes in the intensity of any of these bands. This finding and the failure to find changes in length indicates that there has been no significant amplification of any of the genes at this locus. In some instances (*e.g.*, the 0.3 kb band expected from an EcoRI digestion probed with N1500) the method is quite sensitive, and we could have seen a change as small 50 or 100 base pairs; in other instances the method is less sensitive (*e.g.*, the 10 kb band expected from an XbaI digestion, probed with PP300), and changes much smaller than 1 kb might not have been visible. Nevertheless the data are fairly consistent and suggest that major changes in the primary gene sequence do not occur during activation of the metallothionein genes by these two monofunctional alkylating agents. However, small deletions or insertions or point mutations would not be detectable by this assay.

Toxicity of Sulfur Mustard for S49 Cells. We performed several experiments to analyze the toxicity of sulfur mustard for S49 thymic lymphoma cells. We found that dilution of sulfur mustard in ethanol and the addition of small amounts (~10-25 μl) to large amounts of tissue culture medium (30 ml) gave reasonably reproducible results. The relative survival for one of these experiments is presented in Table 7 and graphed in Figure 4. A concentration of ~1 μM sulfur mustard results in about a 5% survival while ~0.5 μM and ~0.25 μM result in ~20% and 60% survival, respectively. It appears that there is a small shoulder in the log-log plot which suggests that there may be some repair of sulfur mustard damage in these cells.

Generation of Cd^r Colonies After Sulfur Mustard Treatment. We allowed these cells to recover from SM treatment for 5 days in liquid medium and then plated them in either soft agarose without cadmium for relative plating efficiency calculations or soft agarose containing 5 μM cadmium for cadmium resistance measurements (Table 8 and Figure 3). It is clear from these studies that the frequency of Cd^r variants in untreated S49 cells is ~$2.6/10^6$ cells while in survivors of the highest treatment (~1 μM) there is an ~140-fold increase. Thus sulfur mustard is very active in this system.

Analysis of Metallothionein RNA in Cd^r Generated by SM Treatment. We picked colonies from soft agarose and expanded 18 Cd^r variants derived from SM treatment and 5 spontaneous variants (derived from vehicle treatment) and analyzed MT-I and MT-II RNA synthesis in these 23 variants. Table 9 lists the variants studied and the concentration of SM used to generate them. A series of northern blots using probes for MT-I and MT-II (*e.g.*, Figure 6) demonstrated that the great majority of Cd^r lines made at least one and frequently two MTs. In most cases it was easy to determine which lines made MT-I or MT-II; however, in a few lines (*e.g.*, Figure 6, variant EH19) it was difficult to determine if variants were making trace amounts of MT-II. These were scored as +/- (Table 9). It is of interest that the spontaneous lines uniformly (0/5) failed to make MT-I and made MT-II (5/5). In contrast the SM derived lines made either MT-I or MT-II or both. Further, variants from the low treatment group (0.53 μM SM) and those from the high treatment group (1.06 μM SM) showed similar patterns of MT expression. These data establish unequivocally that treatment of S49 cells with SM results in Cd^r variants in which one or both mouse MT genes are activated. In cases in which both MTs are activated (Table 9, *e.g.*, variants EH21, EH31, and EH32) it is unclear whether this results from a single event that affects both of these closely linked genes (Figure 1) or two independent events.

Genomic Changes in SM-generated Cd^r Variants. One possible mechanism which might account for the activation of quiescent genes is changes in genomic structure. To investigate

this possibility we looked for amplifications, deletions, rearrangements and insertions by Southern blotting using convenient restriction endonuclease sites and probes (Figure 1; Table 6). We analyzed both spontaneous variants and those arising following SM treatment for changes in 7 expected fragments covering both the MT-I and MT-II regions of this locus (*e.g.*, Figure 7). In a majority of these 23 Cd^r variants no changes were detected. Thus, the activation of these genes results either from events unrelated to changes in DNA sequence (*e.g.*, changes in chromatin structure, DNA methylation, etc.) or changes too small to be identified by this technique (point mutations, deletions or insertions of only a few base pairs, etc.). However, in 5 variants we found rearrangements (Table 10). All of these rearrangements were detected in the MT-II region and all of the variants expressed MT-II. The degree to which these rearrangements are related to the activation of these genes is uncertain and would require extensive detailed analysis, perhaps entailing the cloning and sequencing of these regions. It is of interest that 3 of the rearranged Cd^r variants come from the vehicle control group and represent 60% of the Cd^r variants in this category. Thus, it is clear that these changes are not dependent on SM treatment. In fact, although 2 Cd^r variants derived from SM treatment show similar changes (Table 10), these might be fortuitous changes unrelated to the action of SM itself. What is apparent from this survey is that no single genomic change is uniquely associated with the activation of MT genes.

Changes in Methylation Patterns in Cd^r Variants Derived From SM Treatment. Since demethylation has been linked to the activation of quiescent genes (9), we undertook a survey of Cd^r lines (Table 9) to determine if demethylation was associated with the activation of the metallothionein genes following treatment with SM. We looked at all of these variants by digesting them with HpaII restriction endonuclease, running an agarose gel, and, following transfer, probing with the appropriate probe. HpaII will cut CCGG sites provided the internal C is unmethylated; thus, this restriction endonuclease distinguishes between methylated and

unmethylated deoxycytidines at CCGG sites (*e.g.*, Fig. 3). We probed blots with either R600 or PP300. These probes look at the 5' and the 3' end of MT-I, respectively. In almost all of these lines we found a series of lower molecular weight fragments. Since only a few fragments would be predicted from each probe (Figure 1), these patterns are indicative of mixed populations of cells within each clone which are demethylated to varying degrees. Almost uniformly every line (with the possible exception of EH27) showed some degree of demethylation using either probe. This finding is of interest because a number of the lines (*e.g.*, EH1, EH2A, EH29, etc.; Table 9) do not synthesize MT-I RNA. Yet these lines do synthesize MT-II RNA. These data suggest that activation of either gene within this region results in changes in methylation patterns. These data also suggest that obligatory demethylation at specific sites is not necessary for the activation of MTs. Rather, conformational changes occurring in the whole region probably are associated with (? require) some demethylation. Although we did not evaluate methylation changes at the MT-II locus because of lack of time, the data from the analysis of the MT-I region indicate that demethylation in this region is intimately associated with activation of the MT genes.

Appearance of Cd^r Variants After Treatment with Half Sulfur Mustard (HSM). As a comparison for our studies with SM, we have also looked at HSM. We treated cells with different concentrations of HSM and seeded them in soft agarose to establish a relative survival curve (Figure 8). The dose at which 50% of the cells survive is approximately 0.1 μM. This value is somewhat surprising since a similar value for SM is 0.3 to 0.4 μM (Fig. 4). After allowing the cells to recover for 5 days, we plated both HSM-treated and untreated cells in agarose alone or agarose containing 5 μM $CdSO_4$. By counting the number of colonies that appeared in the Cd containing agarose and correcting for viability and toxicity (Figure 8), we obtained the data presented in Figure 9. It is clear that treatment of cells with HSM results in the appearance of a large number of cadmium-resistant variants. In fact, at the highest dose

tested (0.375 μM) there was about a 150-fold increase in the number of cadmium-resistant variants per survivor. Thus, on a molar basis SM and HSM have a similar potency in producing Cd^r variants. We did not have time to test these Cd^r variants for metallothionein RNA production. However, because Cd^r variants from both vehicle-treated and SM-treated groups (Table 9) almost invariably made at least one MT, it is likely that HSM induces cadmium resistance by activating the MT genes.

DISCUSSION

Treatment of Cd^s S49 cells with ENU or NA-AAF results in a large increase in Cd^r variants (Table 1). These variants are induced by the action of these alkylating agents rather than selected by the agents from previously existing Cd^r variants in the initial cell population. About two-thirds of these variants make MT-I, MT-II or both RNA. It is of interest that almost 50% of the MT^+ cells make both metallothioneins; further, the metallothioneins, when co-expressed, are usually coordinately regulated as well. Although the mechanism of action of these alkylating agents with respect to gene activation is unknown, it appears that several types of events are involved. This conclusion is based on the fact that at least three classes of MT^+ variants exist ($MT\text{-}I^+$, $MT\text{-}II^+$; and $MT\text{-}I^+/MT\text{-}II^+$). Further, different modes of regulation appear in different variant lines: For example, ENU-6 shows low constitutive expression of MT-I and MT-II while ENU-10 shows high constitutive expression of MT-I and MT-II (Table 3). The finding that almost a third of Cd^r variants are MT^- indicates that other mechanisms in addition to metallothionein synthesis are involved in cadmium resistance. It is possible that these variants may have high thresholds to the transport of cadmium or are able to pump cadmium out of the cell very effectively, but the mechanism of Cd^r resistance in these MT^- cells has not been investigated.

As indicated in the Results section of this report, the DNA methylation patterns in Cd^r variants is distressingly complex (20). Part of this complexity may result from "methylation

drift" (*i.e.*, remethylation of sites initially demethylated during activation or loss of methyl groups subsequent to activation). The fact that no clear, consistent pattern has emerged suggests that these cells do not stringently regulate the methylation pattern of their genomes (see also 21,22). The role of methylation in gene activation is not well understood: Whether loss of methyl groups is a crucial initiating event in the activation of genes or a secondary event which stabilizes the active confirmation of chromatin in unknown (20). These data indicate, however, that precise maintenance of methylation patterns is not necessary for continued expression and suggest that demethylation of a certain percentage of sites within a given region may be sufficient to maintain the active state (see ref. 23). It is worth noting that the methylation patterns in these cells were obtained many generations after the initial activating event(s). This lag is necessary in order to grow up sufficient quantities of cells (typically 1-3 x 10^7 cells) to prepare nucleic acids for analysis.

These results also demonstrate that in addition to the many deleterious effects of alkylating agents on gene expression and survival in some instances they can activate quiescent, unexpressed genes. Further, the fact that there is coactivation of both MT-I and MT-II in a significant portion of cases suggests that alkylating agents may be capable of activating genes involved in the control of genetic programs. It is well to note that there is no evidence that alkylating agents act <u>cis</u> to MT-I and/or MT-II; rather, the effects of these agents on MT-I and MT-II may result from activation of a <u>trans</u> factor or factors which regulate the expression of these genes. Given the tools presently available, it is not possible to evaluate this problem directly; however, the identification of a pathway in which alkylating agents can activate genes and gene families provides evidence for an additional type of cellular response to these toxic agents.

It is clear from our previous data (1) and our present data (Results section) that ultraviolet radiation and two monofunctional alkylating agents result in large increases in Cd^r

colonies in S49 cells, many of which make MT-I, MT-II, or both. Changes in DNA methylation are associated with activation of the MT genes, but no simple pattern of methylation change can be directly related to the activation of these genes. An extension of this analysis (Figure 1, Table 6) in which we looked for insertions, deletions, rearrangements, or amplifications associated with the activation of these genes, failed to yield any changes. It is possible that activation is the result of point mutations or small deletions or insertions which the present methodology cannot detect. In theory it is possible to use probes designed to detect mismatches to analyze these events (24,25). This method has been used successfully to detect ras mutations in nucleic acids from patients with colon cancers. However, unlike that study in which only about 300 base pairs had to be examined, in the present study there is no indication where such mutations might exist. Thus, at the present time, it seems inappropriate to apply this methodology. Another way in which these monofunctional alkylating agents might activate the MT genes is by inducing changes in chromatin structure which are passed from one generation to the next. It is well known that during development the activation of genes is accompanied by changes in chromatin structure as well as DNA methylation. Although it is possible to analyze changes in chromatin structure (*e.g.*, analysis of DNase I or staphylococcal nuclease hypersensitive sites), at present there are no well developed strategies for looking at the mechanisms of these changes. Thus although it is clear that all of the above agents can activate these two quiescent genes, the analysis of the mechanism of activation will prove difficult.

We have also found that SM is very active in the S49/metallothionein system. The toxicity of SM for S49 cells is somewhat greater than that observed for Hela cells (Table 7; ref. 26). Following treatment with SM, there is a large increase in Cd^r variants (Figure 5). Our data indicate that some survivors of SM treatment are cadmium-resistant and that a majority of these make either MT-I, MT-II, or both (Table 9). Major insertions, deletions or

rearrangements in the MT region of the genome do not appear to be involved in the activation process (Tables 6 & 10); however, focal changes such as the deletion or insertion of only a few bases would not be detectable by our techniques. Thus, it is not clear whether mutation plays a role in this process or not. Although when we first proposed this work, there was no convenient way to analyze MT^+ variants for such changes, the development of polymerase chain reaction techniques followed by sequencing now make this problem approachable (27,28). Many of the Cd^r variants express both MT-I and MT-II. Whether the mechanism is mutational or non-mutational any complete explanation of the effects of SM must take into account the simultaneous activation of two genes. It is conceivable, of course, that a single mutation at a regulatory locus or in the MT gene region itself might effect both genes simultaneously or that induction of changes in chromatin structure (23) or methylation patterns could effect both genes as well.

The changes in methylation patterns which accompany the activation of the MT genes are complex and suggest that although the lines were clonally derived there has been some drift in methylation patterns with expansion. Such drift is known to occur in cell culture (21,22). Neither we nor other groups have an explanation for this apparent paradox: On the one hand methylation is proposed to be important in the regulation of genes, but on the other there is a drift in methylation patterns with time in culture. It has been postulated either that only a percentage of the sites needs to be demethylated or that there are a few crucial sites of demethylation related to gene expression and the rest are irrelevant. Neither or these explanations has been tested rigorously.

We found that half sulfur mustard was also effective in producing cadmium-resistant variants. Surprisingly, in our hands HSM (2-chloroethyl-ethyl sulfide) and SM were approximately equally toxic and equally effective in producing Cd^r variants. In general, others have found SM to be more toxic than HSM (26). Whether these differences relate to some

unusual aspect of the way we have treated cells or the S49 cells themselves is unknown. However, perhaps more important than the quantitative results is the observation that both agents can produce Cd^r variants. This finding demonstrates that DNA cross-linking is not necessary for the action of the mustards in this system.

In the living mammal, the actions of the mustards are complex, and their analysis is complicated by the need to study many biochemical, physiologic, and toxicologic changes simultaneously. Although the S49 mouse thymic lymphomic system is too simple to be a model for the whole spectrum of SM actions, it is a useful way to study the genomic effects of this agent. It should be possible, for example, to examine the mechanisms of activation by determining if there are point mutations in the MT gene region (using the polymerase chain reaction) or if the activation of this locus results from changes from trans acting factors, etc. Thus, the system lends itself not only to understanding practical problems in the toxicology of SM but also to understanding the molecular biology of DNA damage.

REFERENCES

1. Roberts, J.J., Pascoe, J.M., Plant, J.E., Sturrock, J.E., and Crathron, A.R. (1971) Quantitative aspects of the repair of alkylated DNA in cultured mammalian cells. *Chem. Biol. Interactions* 3, 29-47.
2. Miller, J.A. and Miller, E.C. (1977) Ultimate chemical carcinogens as reactive mutagenic electrophiles. In *Origins of Human Cancer* (H.H. Hiatt, J.D. Watson, and J.A. Winston, eds.). Cold Spring Harbor Laboratory, pp. 605-628.
3. Roberts, J.J., Pera, M.F., and Rawlings, C.J. (1982) The role of DNA repair in the recovery of mammalian cells from cis-diamminodichloroplatinum (II) (cisplatin)-induced DNA damage. In *DNA Repair, Chromosome Alterations and Chromatin Structure* (A.T. Natarajan, G. Obe, and H. Altmann, eds.) Elsevier Biomedical, Amsterdam, pp. 223-246.
4. Farber, E. (1984) The multistep nature of cancer development. *Cancer Res.* 44, 4217-4233.
5. Sell, S. and Warren, B. (1982) *Human Cancer Markers*, Humana Press, Clifton, New Jersey.
6. Barbacid, M. (1987) ras Genes. *Ann. Rev. Biochem.* 56, 779-827.
7. Malling, H.V. and Wassom, J.S. (1977) Action of mutagenic agents. In *Handbook of Teratology* (J.G. Wilson and F.C. Fraser, eds.), Plenum Press, New York, pp. 99-152.
8. Lieberman, M.W., Beach, L.R. and Palmiter, R.D. (1983) Ultraviolet radiation-induced metallothionein-I gene activation is associated with extensive DNA demethylation. *Cell* 35, 207-214.
9. Karin, M. (1985) Metallothioneins: Protein in search of function. *Cell* 41, 9-10.
10. Compere, S.J. and Palmiter, R.D. (1981) DNA methylation controls the inducibility of the mouse metallothionein-I gene in lymphoid cells. *Cell* 25, 233-240.

11. Mayo, K.E. and Palmiter, R.D. (1981) Glucocorticoid regulation of metallothionein-I mRNA synthesis in cultured mouse cells. *J. Biol. Chem.* 256, 2621-2624.

12. Searle, P.F., Davison, B.L., Stewart, G.W., Wilkie, T.M., Norstedt, G. and Palmiter, R.D. (1984) Regulation, linkage and sequence of mouse metallothionein I and II genes. *Mol. Cell. Biol.* 4, 1221-1230.

13. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Isolation of total cellular RNA. In *Molecular Cloning - A Laboratory Manual.* Cold Spring Harbor Laboratory Press, New York, p. 196.

14. White, B.A. and Bancroft, F.C. (1982) Cytoplasmic dot hybridization. *J. Biol. Chem.* 257, 8569-8572.

15. Hu, N.T. and Messing, J. (1982) The making of strand-specific M13 probes. *Gene* 17, 271-277.

16. Brown, D.M., Frampton, J., Goelet, P. and Karn, J. (1982) Sensitive detection of RNA using strand-specific M13 probes. *Gene* 20, 139-144.

17. Thomas, P.S. (1980) Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA* 77, 5201-5205.

18. Durnam, D.M., Perrin, F., Gannon, F., and Palmiter, R.D. (1980) Isolation and characterization of the mouse metallothionein-I gene. *Proc. Natl. Acad. Sci. USA* 77, 6511-6515.

19. Glanville, N., Durnam, D.M., and Palmiter, R.D. (1981) Structure of the mouse metallothionein-I gene and its RNA. *Nature* 292, 267-269.

20. Doerfler, W. (1983) DNA methylation and gene activity. In *Annual Reviews in Biochemistry* (Snell, E.E., Boyer, P.D., Meister, A. and Richardson, C.C., eds.), Annual Reviews, Inc., Palo Alto, California, Vol. 52, pp. 93-124.

21. Reis, R.J.S. and Goldstein, S. (1982) Interclonal variation in methylation patterns for

expressed and non-expressed genes. *Nucleic Acids Res.* 10, 4293-4304.

22. Wilson, W.L. and Jones, P.A. (1983) DNA methylation decreases in aging but not immortal cells. *Science* 230, 1055-1057.

23. Orlofsky, A. and Chasin, L.A. (1985) A domain of methylation change at the albumin locus in rat hepatoma cell variants. *Mol. Cell. Biol.* 5, 214-225.

24. Bos, J.L., Fearon, E.R., Hamilton, S.R., Verlaan-de Vries, M., van Boom, J.H., van der Eb, A.J., and Vogelstein, B. (1987) Prevalence of ras gene mutations in human colorectal cancers. *Nature* 327, 293-297.

25. Forrester, K., Almoguera, C., Han, K., Grizzle, W.E., and Perucho, M. (1987) Detection of high incidence of K-ras oncogenes during human colon tumorigenesis. *Nature* 327, 298-303.

26. Craethorn, A.R., and Roberts, J.J. (1966) Mechanism of the cytotoxic action of alkylating agents in mammalian cells and evidence for the removal of alkylated groups from deoxyribonucleic acid. *Nature* 211, 150-153.

27. Wong, C., Dowling, C.E., Saiki, R.K., Higuchi, R.G., Erlich, H.A., and Kazazian, H.H., Jr. (1987) Characterization of ß-thalassemia mutations using direct genomic sequencing of amplified single copy DNA. *Nature* 330, 384-386.

28. Wrischnik, L.A., Higuchi, R.G., Stoneking, M., Erlich, H.A., Arnheim, N., and Wilson, A.C. (1987) Length mutations in human mitochondrial DNA: Direct sequencing of enzymatically amplified DNA. *Nucleic Acids Research* 15, 529-542.

Table 1. Survival and Appearance of Cd^r in S49 Cells Treated with Ethylnitrosourea and N-acetoxy-2-acetylaminofluorene

Compound	Dose	Survival[a,b]	Cd^r Colonies per 10^6 Viable Cells[a]
0	0	1.0 ± 0.01	30 ± 2.2
ENU	1 mM	0.76 ± 0.04	287 ± 13.9
ENU	2 mM	0.29 ± 0.02	499 ± 28.9
ENU	3 mM	0.034 ± 0.01	822 ± 72.0
NAAF	5 μM	0.95 ± 0.02	108 ± 13.2
NAAF	10 μM	0.75 ± 0.01	266 ± 18.1
NAAF	15 μM	0.30 ± 0.01	376 ± 23.7

[a]Results are the average of five plates ± SEM.
[b]Values are normalized to the 0 treatment group; absolute plating efficiency was ~80%.

Table 2. MT-I and MT-II RNA Expression in Cd^r Variants of S49 Cells[a]

Number of Variants Examined	$MT\text{-}I^+$	$MT\text{-}II^+$	$MT\text{-}I^+/MT\text{-}II^+$	MT^0
47	9	7	15	16
	19%	15%	32%	34%

[a]Based on RNA dot blotting (see Materials and Methods).

Table 3. Qualtitation of Steady State MT RNA Levels

	MT-I RNA (Molecules/Cell)[1]			MT-II RNA (Molecules/Cell)[1]		
	No addition	Dex[2]	Cd[3]	No addition	Dex[2]	Cd[3]
S49	<2	<2	<2	<1	<1	<1
ENU-5	<6	<6	20	140	150	4100
ENU-6	100	<6	70	260	70	1900
ENU-7	70	70	570	190	160	1100
ENU-10	770	1900	2500	860	1500	1200
AAF-1	110	210	4500	<1	<1	<1
AAF-4	280	440	1900	700	900	6000
AAF-6	230	190	2400	380	700	6400
AAF-7	60	110	630	240	190	880

[1]Determined from FNA dot blots with SP6 transcripts as standards (see Materials and Methods)
[2]100 nM dexamethasone (6 hr)
[3]20 μM Cd (6 hr)

Table 4. Study of Selection vs. Induction of Cd-Resistant Variants

Treatment	CFE[a]	Cd-resistant[b] colonies per 4 x 10^5 cells	Cd-resistant colonies per 10^6 viable cells
1% DMSO	0.87 ± 0.04	16 ± 2	46 ± 6
2 mM ENU (in 1% DMSO)	0.21 ± 0.03	4 ± 1	48 ± 16
15 μM NA-AAF (in 1% DMSO)	0.24 ± 0.04	5 ± 1	51 ± 17

[a]Colony forming efficiency; 300 cells were seeded in soft agar without Cd immediately after treatment. These and all other data in Tables 4 and 5 are the average of 5 dishes ± SEM.
[b]4 x 10^5 cells were seeded in soft agarose containing 5 μM $CdSO_4$ immediately after treatment.

Table 5. Growth of Cd-Sensitive and Cd-Resistant Cells After Carcinogen Treatment

Group	Treatment	CFE with Cd[b]	CFE without Cd[b]	Fraction Cd-resistant cells
1[a]	1% DMSO	0.29 ± 0.02	0.89 ± 0.02	0.33 ± 2
1	2 mM ENU in 1% DMSO	0.30 ± 0.01	0.87 ± 0.03	0.34 ± 2
2	1% DMSO	0.19 ± 0.02	0.87 ± 0.04	0.22 ± 3
2	10 μ NA-AAF	0.23 ± 0.03	0.88 ± 0.02	0.26 ± 3

[a]Group 1 is a mixture of S49 (Cd-sensitive) cells and a Cd-resistant line derived from ENU treatment; group 2 is a mixture of S49 cells and a Cd-resistant line derived from NA-AAF treatment.
[b]200 cells per dish; 5 dishes (soft agarose) were used for each point; data are ± SEM; cells were assayed 5 days after treatment to allow any selective advantage to appear prior to seeding.

Table 6. Fragment Length from Southern Blot Analysis of Parental (MT^-) and MT^+ Variants Induced by ENU and NAAF

Gene	Expected Band* in kb	Enzyme**	Probe**
MT-I	3.8	EcoRI	KB600
MT-I	10.0	XbaI	PP300
MT-II	9.0, 0.7, 0.3	EcoRI	N1500
MT-II	4.0, 2.1	PstI	N1500
MT-I/MT-II	15.0	BamHI	KB600

*In all cases the expected and observed fragment lengths were the same.
**See Figure 1 for map.

Table 7. Survival of S49 Cells Following Sulfur Mustard Treatment*

Final SM Concentration	Soft Agarose Colonies/Dish	Cd^r colonies/ Relative Survival**
0	373, 383	1.0
0.26 μM	219, 229	0.59
0.53 μM	100, 116	0.28
1.06 μM	14, 26	0.05

*See Materials and Methods and Legend to Figure 4 for details. Cells were seeded in duplicate in soft agarose.
**Computed by averaging the values in column 2 and dividing by the value for 0 treatment.

Table 8. Appearance of Cd^r Following Sulfur Mustard Treatment*

Final SM Concentration	Relative Plating Efficency	Cd^r Colonies/ 10^6 Cells Plated**	Cd^r Colonies/ 10^6 Survivors***
0	1.0	2.6	2.6
0.26 μM	1.10	6.9	11.7
0.53 μM	1.14	10.5	37.5
1.06 μM	1.02	18.5	370.0

*See Materials and Methods and Legend to Table 7 for details.
**Corrected for plating efficiency.
***Corrected for relative survival (Table 7).

Table 9. Metallothionein RNA Synthesis in Cd^r Variants Derived From Sulfur Mustard Treatment*

Treatment	Line	MT-I	MT-II
None (parental cell type; Cd^s)	S49	-	-
Vehicle only (spontaneous variants)	EH1	-	+
	EH2A	-	+
	EH3A	-	+
	EH4A	-	+
	EH5	-	+
0.53 μM Sulfur Mustard	EH19	+	+/-
	EH21	+	+
	EH22	-	+
	EH23	+	+/-
	EH24	+	+/-
	EH25	+	+
	EH26	+	+
	EH27	-	+
	EH29	-	+
	EH31	+	+
	EH32	+	+
	EH34	+	+
	EH35	-	+
1.06 μM Sulfur Mustard	EH36	-	+
	EH37	-	+
	EH41	-	+/-
	EH43	+	+
	EH50	+	+

*Determined from northern blots, *e.g.*, Figure 6.

Table 10. Rearrangements in Cd^r Variants Derived from SM Treatment

Cell Line	Treatment Group	Phenotype	Band Length (Kb)	Enzyme/Probe
EH1	Vehicle only	MT-I$^-$/MT-II$^+$	1.2	EcoRI/N1500
EH3A	Vehicle only	MT-I$^-$/MT-II$^+$	~1.0 (weak)	PstI/N1500
EH4A	Vehicle only	MT-I$^-$/MT-II$^+$	3.0, 2.5 3.5	EcoRI/N1500 PstI/N1500
EH21	0.53 μM SM	MT-I$^+$/MT-II$^+$	3.7	PstI/N1500
EH50	1.06 μM SM	MT-I$^+$/MT-II$^+$	20.0 3.0	EcoRI/N1500 PstI/N1500

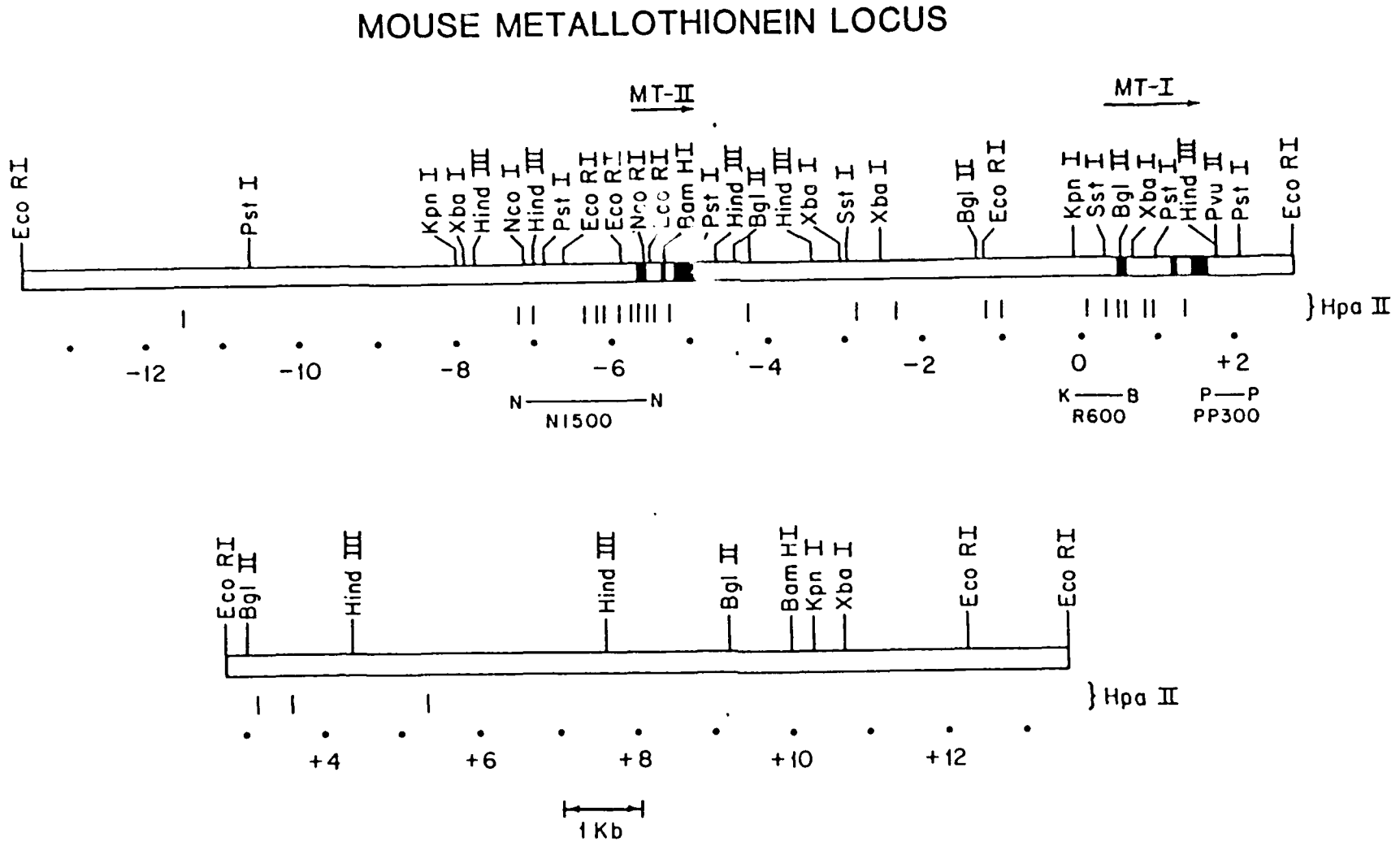

Figure 1: Organization of the mouse metallothionein locus. The black rectangles represent exons of MT-II (5') and MT-I. Three letter codes above the locus represent known restriction sites. Short vertical lines below the locus represent known sites of HpaII cutting, and dots beneath these are 1 kb markers. Beneath the gene the N1500, R600 and PP300 probes are shown.

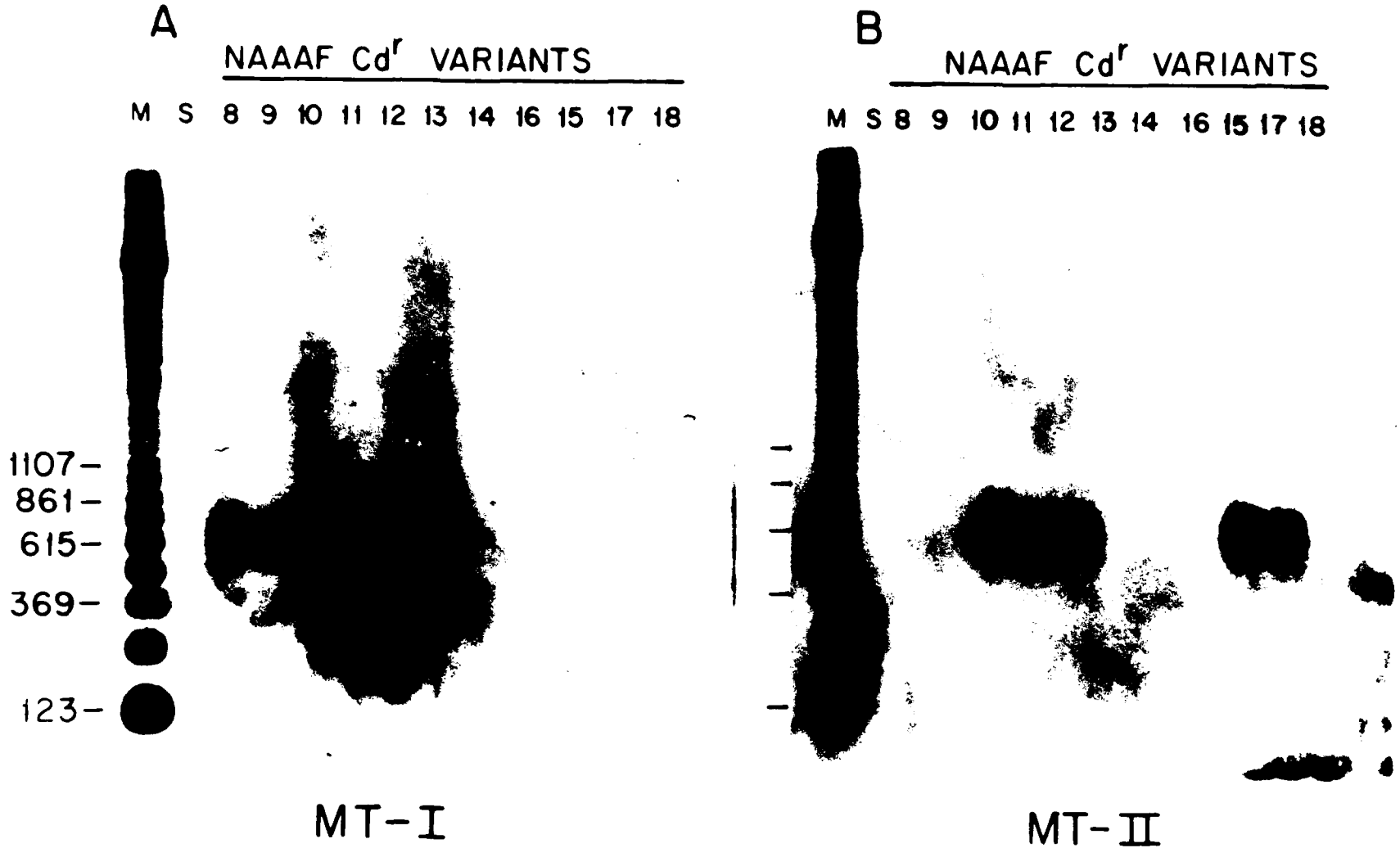

Figure 2: Northern blot analysis of metallothionein synthesis in S49 cadmium-resistant variants. RNA (10 μg) from S49 cells (lane S) and cadmium-resistant variants was denatured with glyoxal and run on a 1% agarose-glyoxal gel (Materials and Methods). After blotting to nitrocellulose, the gels were probed with a probe for MT-I (Figure 2A) or MT-II (Figure 2B) (see Materials and Methods). The numbers to the left represent molecular weight markers in kbp.

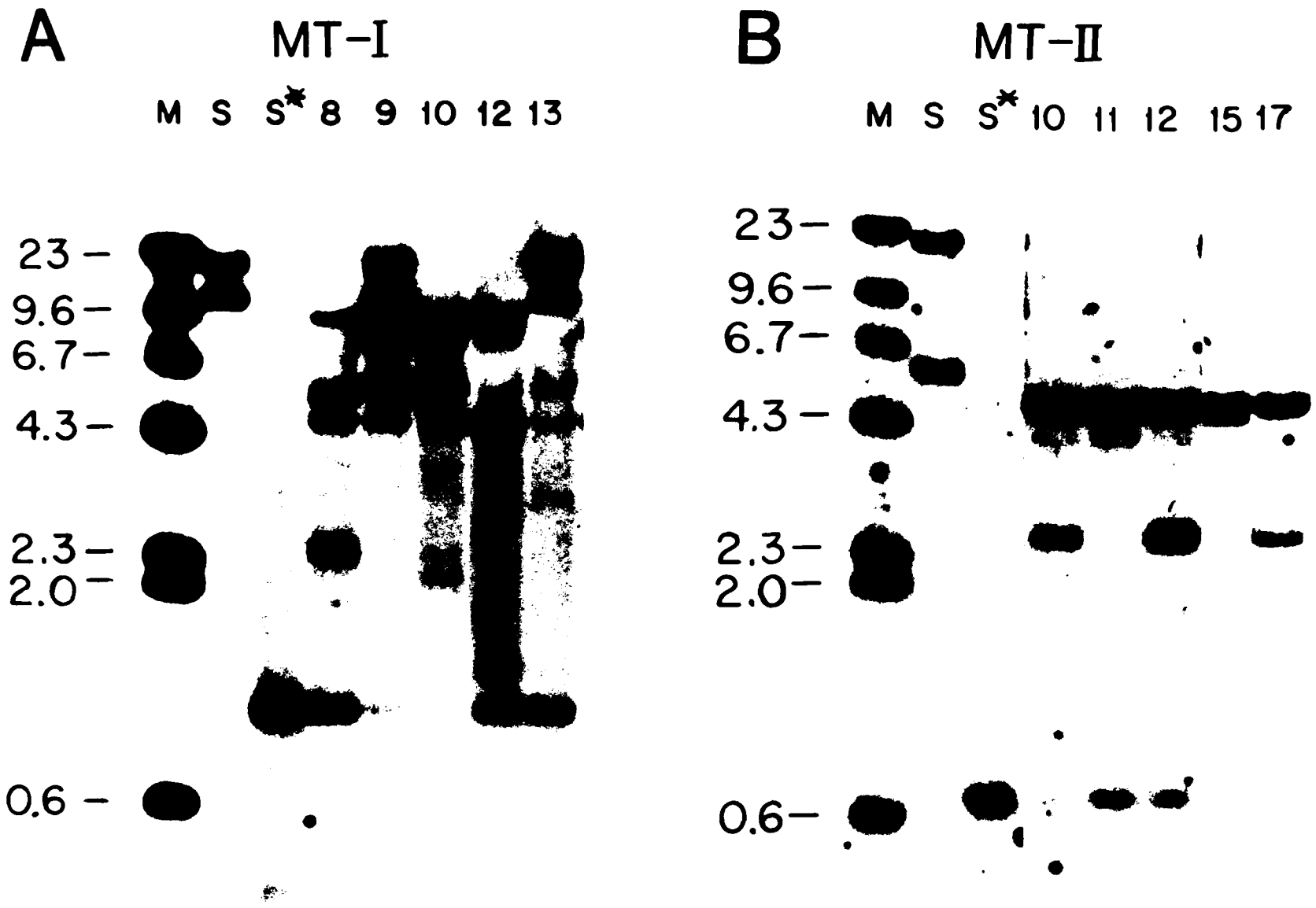

Figure 3: Analysis of demethylation in lines synthesizing MT-I (Figure 3A) and MT-II (Figure 3B). 10 µg of DNA was cut with HpaII, electrophoresed on 1% agarose gels, blotted to nitrocellulose and hybridized with ^{32}P-labeled R600 (3A) or N1500 (Figure 3B). Numbers to the left of the blots are molecular weight markers in kbp (lane M). Lane S is a digest of Cd^s S49 cells with HpaII, while lane S* is a digest of these cells with MspI. This enzyme cuts CCGG sequences whether or not the internal C is methylated and serves as a standard. Other numbers refer to cadmium-resistant variants known to make MT-I (Figure 3A) or MT-II (Figure 3B).

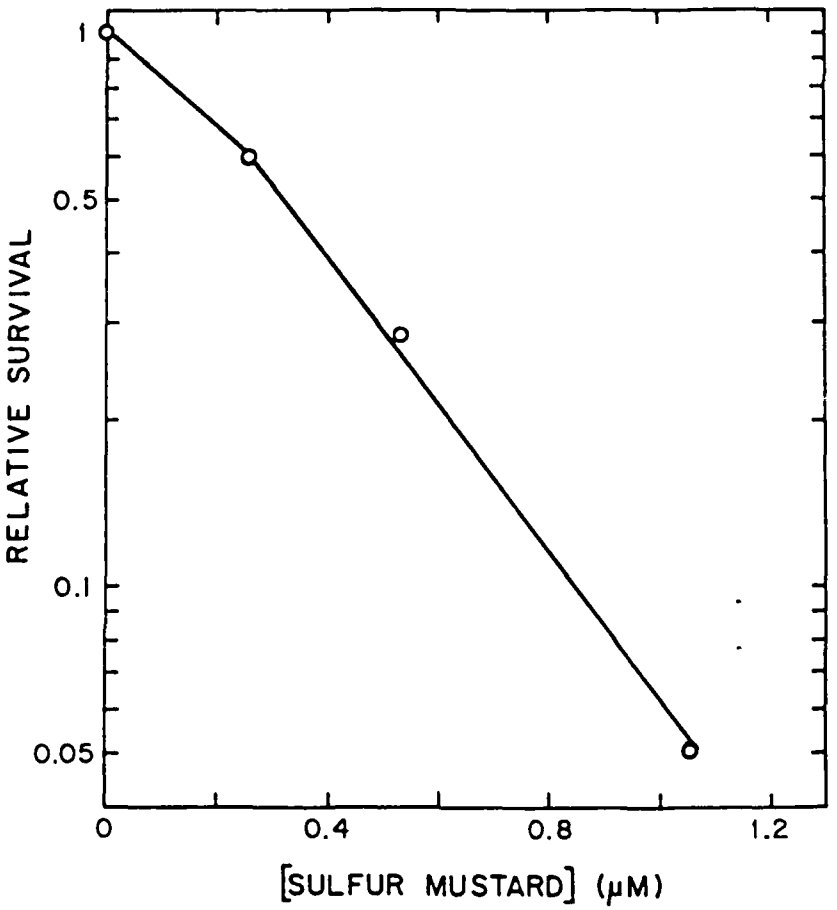

Figure 4. Toxicity of sulfur mustard for S49 mouse lymphoma cells. S49 cells were treated in Ham's F12 medium with 10% fetal calf serum at the indicated molarities of sulfur mustard for three hours at 37°C. Following treatment, cells were plated at 400 cells per 100 mm dish in 0.5% soft agarose. Colonies were counted at 14 days.

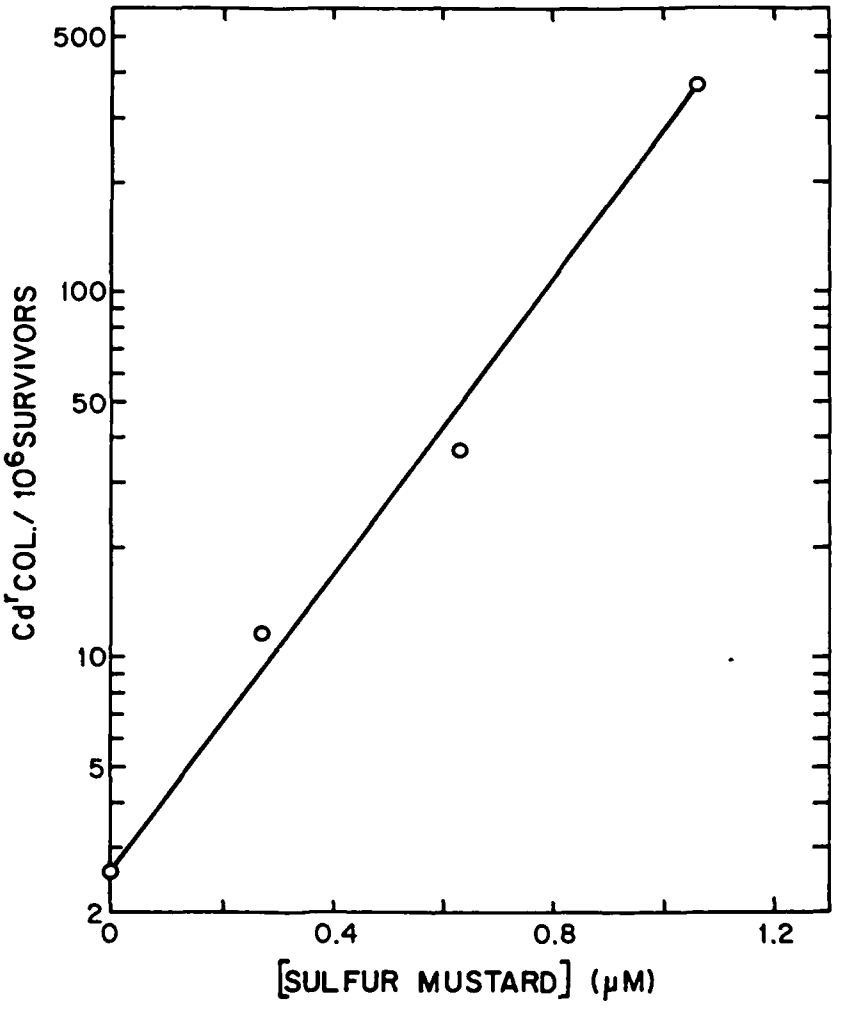

Figure 5. Appearance of Cd^r variants following sulfur mustard treatment. Cells were treated with sulfur mustard as described in the text and legend to Figure 4 and allowed to recover for 5 days in liquid medium. They were then plated at 10^6 cells per 100 mm petri dish in 0.5% soft agarose containing 5 μM Cd (5 dishes per dose). Similar aliquots for plating efficiency were plated at 200 to 1,000 cells per dish in agarose without Cd. the number of Cd^r colonies in each dish was counted at 14 days and corrected for relative survival (Figure 4).

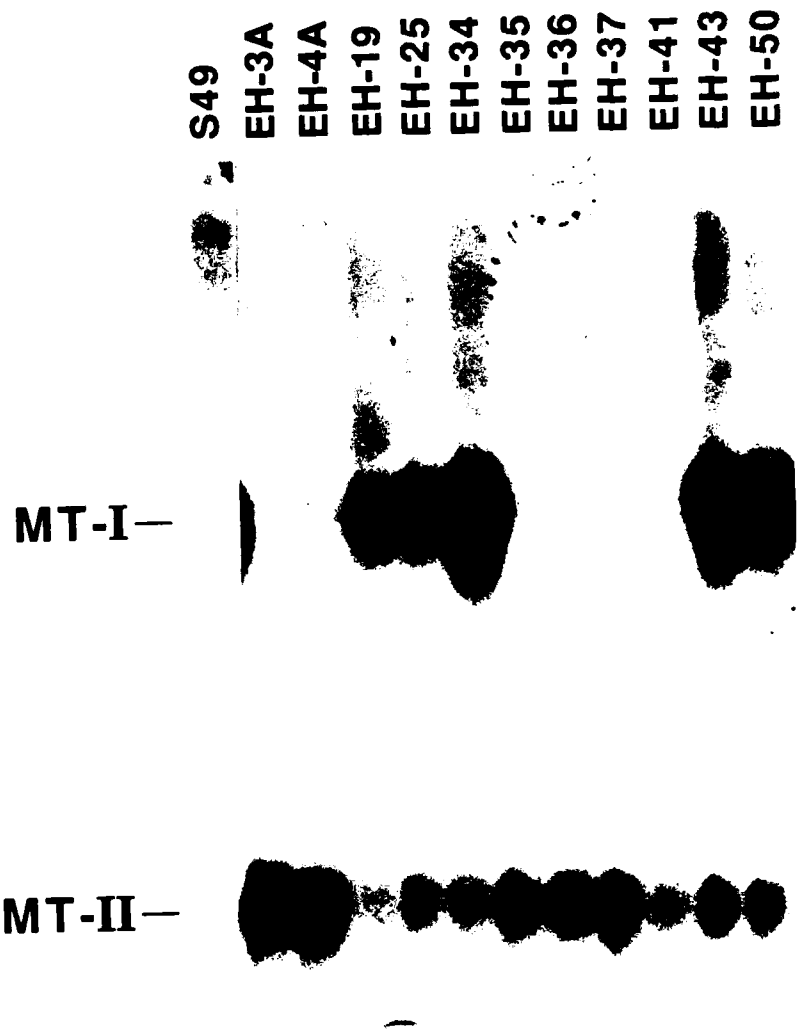

Figure 6. Analysis of steady state MT-I and MT-II RNA levels by northern analysis. Ten micrograms of total cellular RNA was used. S49 cells are parental Cd^s cells. Other lines (EH3A, etc.) are Cd^r variants derived from SM treatment. The figure is a composite of two blots.

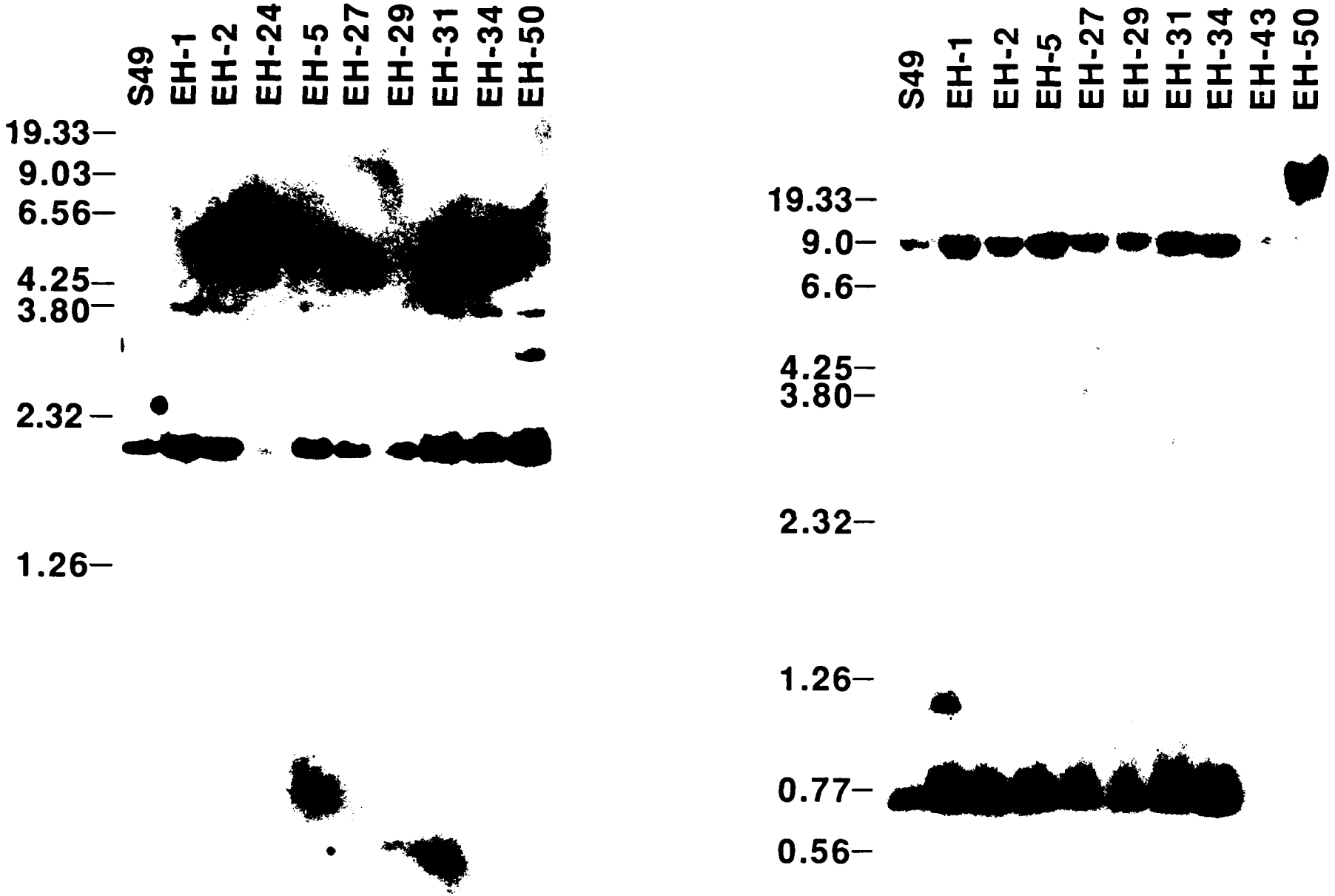

Figure 7. Identification of changes in genomic structure by Southern blotting. DNAs (10 μg) from S49 cells and Cd^r variants derived from SM treatment were cut with PstI (left) or EcoRI (right) and probed with N1500 (Figure 1).

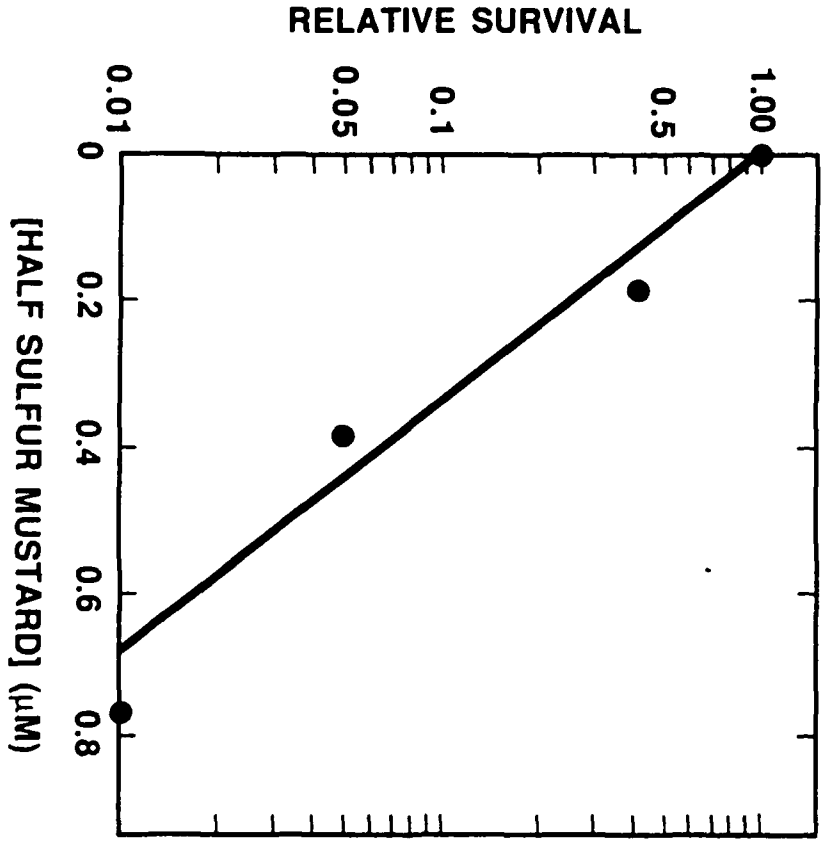

Figure 8. Toxicity of half sulfur mustard for S49 mouse lymphoma cells. S49 cells were treated in Ham's F12 medium with 10% fetal calf serum at the indicated molarities of half sulfur mustard (in ethanol) for three hours at 37°C. Following treatment, cells were plated at 400 cells per 100 mm dish in 0.5% soft agarose. Colonies were counted at 14 days.

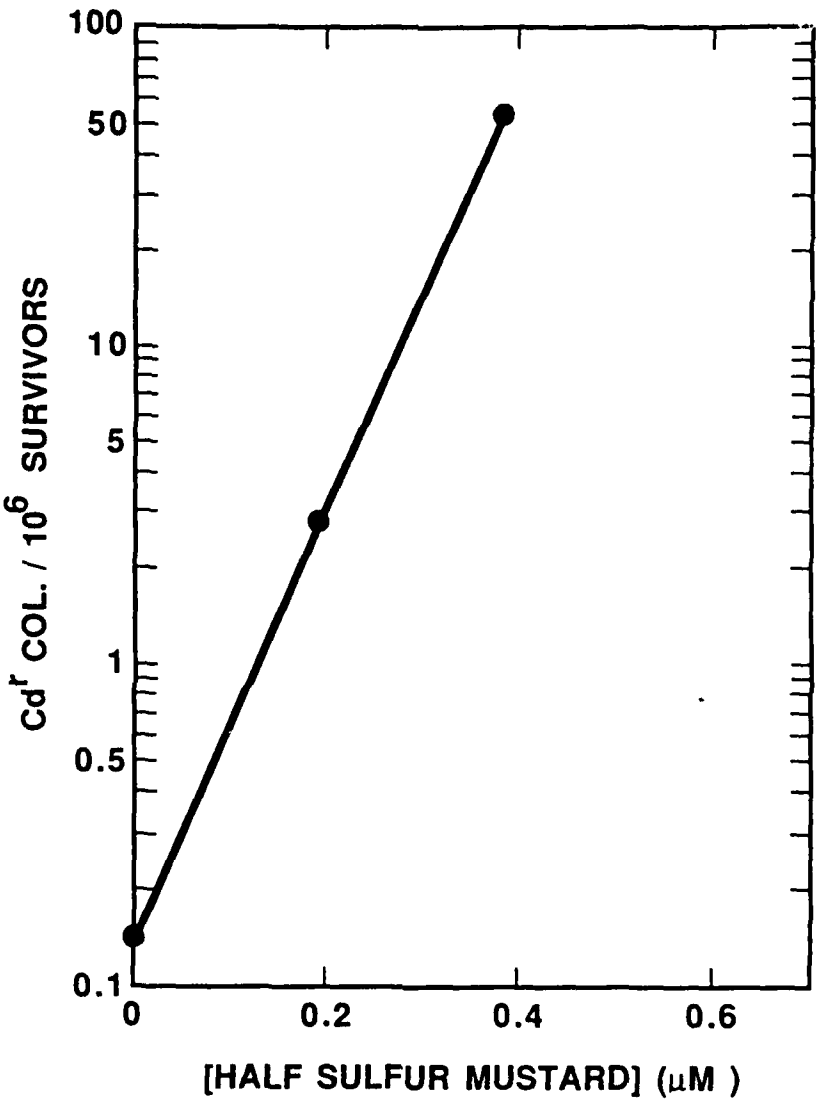

Figure 9. Appearance of Cd^r variants following half sulfur mustard treatment. Cells were treated with half sulfur mustard as described in the text and legend to Figure 3 and allowed to recover for 5 days in liquid medium. They were then plated at 10^6 cells per 100 mm petri dish in 0.5% soft agarose containing 5 μM Cd (5 dishes per dose). Similar aliquots for plating efficiency were plated at 200 to 1,000 cells per dish in agarose without Cd. The number of Cd^r colonies in each dish was counted at 14 days and corrected for relative survival (Figure 8).